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Sorghum bicolor's transcriptome response to dehydration, high salinity and ABA

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Abstract

Genome wide changes in gene expression were monitored in the drought tolerant C4 cereal *Sorghum bicolor*, following exposure of seedlings to high salinity (150 mM NaCl), osmotic stress (20% polyethylene glycol) or abscisic acid (125 μM ABA). A sorghum cDNA microarray providing data on 12 982 unique gene clusters was used to examine gene expression in roots and shoots at 3- and 27-h post-treatment. Expression of ~2200 genes, including 174 genes with currently unknown functions, of which a subset appear unique to monocots and/or sorghum, was altered in response to dehydration, high salinity or ABA. The modulated sorghum genes had homology to proteins involved in regulation, growth, transport, membrane/protein turnover/repair, metabolism, dehydration protection, reactive oxygen scavenging, and plant defense. Real-time PCR was used to quantify changes in relative mRNA abundance for 333 genes that responded to ABA, NaCl or osmotic stress. Osmotic stress inducible sorghum genes identified for the first time included a beta-expansin expressed in shoots, actin depolymerization factor, inositol-3-phosphate synthase, a non-C4 NADP-malic enzyme, oleosin, and three genes homologous to 9-*cis*-epoxycarotenoid dioxygenase that may be involved in ABA biosynthesis. Analysis of response profiles demonstrated the existence of a complex gene regulatory network that differentially modulates gene expression in a tissue- and kinetic-specific manner in response to ABA, high salinity and water deficit. Modulation of genes involved in signal transduction, chromatin structure, transcription, translation and RNA metabolism contributes to sorghum's overlapping but nonetheless distinct responses to ABA, high salinity, and osmotic stress. Overall, this study provides a foundation of information on sorghum's osmotic stress responsive gene complement that will accelerate follow up biochemical, QTL and comparative studies.

Introduction

Crop productivity is limited to a large extent by saline soils, drought and nutrient deficiency (Boyer, 1982). The demand for water from non-agricultural sectors is increasing, indicating that there will be little new opportunity to increase crop productivity through irrigation (Johnson *et al.*, 2001; Gleick,

2003). In addition, nearly all land suitable for agriculture is currently in use or protected to preserve habitats, and saline soils are prevalent in many regions due to years of irrigation. Therefore, to meet the demands of an increasing world population, the adaptation and yield of crops grown in saline soils and in environments subject to periodic drought needs to increase significantly (Briggs, 1998; Khush, 1999).

Plants adapt to water deficit and saline soils using a wide range of responses (Hsiao, 1973; Ludlow and Muchow, 1990; Blum, 1996; Bray, 1997; Hasegawa *et al*., 2000). In crop plants, variation in root system and leaf canopy architecture, type of photosynthesis (C3/C4/CAM), phenology, and stomatal/leaf cuticle characteristics contribute significantly to differences in adaptation to water limited environments. Stress-inducible responses such as the accumulation of compatible solutes (Koster and Leopold, 1988; Delauney and Verma, 1993; Yoshiba *et al*., 1995; Garg *et al*., 2002; Rontein *et al*., 2002) and dehydrins (Dure, 1993; Close, 1996), stomatal closure (Price *et al*., 2002), differential root growth (Sharp *et al*., 1988), and delayed leaf senescence (Rosenow *et al*., 1996) are also important contributors to plant adaptation and crop yield. The genetic basis of adaptation to adverse environments is complex, consistent with the large number of developmental, biochemical, and physiological responses plants deploy in response to various abiotic constraints. Moreover, adaptation to specific environments varies within the germplasm of a crop (Lee, 1998). QTL studies have identified loci for cold tolerance in barley (Ismail *et al*., 1999), and drought tolerance in maize (Tuberosa *et al*., 2002) and sorghum (Tuinstra *et al*., 1997; Crasta *et al*., 1999). However, QTL studies identify loci that account for only a portion of the genetic variance in adaptation between genotypes consistent with our understanding that mechanisms of adaptation and response to adverse environments are complex.

Recent genome wide analyses of mRNA abundance showed that expression of 5–30% of the genes assayed was modulated by abiotic stress (Ozturk *et al*., 2002; Oono *et al*., 2003; Rabbani *et al*., 2003; Wang *et al*., 2003). In some cases, differential regulation of specific genes and pathways has been associated with improved adaptation of crop genotypes (Zhang *et al*., 2004). New information about signal transduction pathways that modulate gene expression in response to dehydration, cold, and high salt has become available in recent years (Zhu, 2001a; Xiong *et al*., 2002; Shinozaki *et al*., 2003). Moreover, an in depth analysis of genes regulated by abscisic acid (ABA), a key hormone that modulates plant responses to osmotic stress, has been carried out for Arabidopsis (Hoth *et al*., 2002) and rice (Rabbani *et al*., 2003). The ABA signal transduction pathway has also been clarified (Finkelstein *et al*., 2002; Himmelbach *et al*., 2003). These advances provide a solid foundation for interpreting genome wide changes in gene expression induced by ABA and osmotic stress.

The Poaceae family includes most of the agriculturally and economically important cereals including rice, maize, wheat, sorghum, rye, barley, oat and millets. Among the grasses, sorghum (*Sorghum bicolor* (L.) Moench) is especially well adapted to hot/dry adverse environments (Doggett, 1988). The adaptation is based in part on biochemical and structural features such as C4 photosynthesis, deep root architecture, and a thick waxy cuticle that improves water use efficiency. Sorghum also has a complement of genes and alleles that help match plant phenology with available water supply (Morgan *et al*., 2002), mediate osmotic adjustment (Ludlow *et al*., 1990; Wood *et al*., 1996), and in some genotypes an unusual ability to 'stay green' during post-anthesis drought (Rosenow, 1983). The loci controlling some of these traits have been mapped and are currently under investigation (Tuinstra *et al*., 1997; Crasta *et al*., 1999; Xu *et al*., 2000).

To facilitate the identification and isolation of sorghum genes involved in adaptation to adverse environments, genome resources enabling analysis of sorghum trait loci and gene expression have been developed (Klein *et al*., 2000, 2003; Mullet *et al*., 2001). A high-density sorghum genetic map with over 3000 DNA markers has been constructed (Menz *et al*., 2002) and integrated with an emerging BAC-based sorghum physical map (Klein *et al*., 2000). DNA markers and sequence-based information are being used to align the sorghum genome map with the nearly complete rice genome sequence and maps of other grasses (Ming *et al*., 1998; Draye *et al*., 2001; Klein *et al*., 2003). In addition, a sorghum EST project has collected over 200 000 sequences from cDNA libraries derived from diverse tissues and

treatments (http://fungen.org/Sorghum.htm). Clustering of 55 783 3′ ESTs identified 16 801 unique transcripts among the sorghum EST collection (Pratt, *et al*., 2005). A set of cDNA clones representing these unique transcripts is currently being used to collect a compendium of microarray-based expression profiles for sorghum as a first step towards understanding the sorghum transcriptome (Salzman *et al*., 2005; results herein). Therefore, researchers are now well positioned to apply genome wide approaches to investigate the molecular basis of sorghum's unusual adaptation to adverse environments. In this study, the construction and utilization of a sorghum cDNA microarray was validated and a baseline of information collected on sorghum genes that respond to dehydration, high salinity and ABA.

Materials and methods

Plant growth and osmotic stress treatments in sorghum

Seeds of *Sorghum bicolor* cultivar BTx623 were sterilized and germinated for 3 days on germination paper (Anchor Paper, St. Paul, MN) before being transferred to custom hydroponic tanks holding ~40 plants each. Seedlings were grown hydroponically under constant aeration in 0.5 × Hoagland's nutrient solution in a growth chamber at 31 °C day, 22 °C night temperature. Day length was 12 h, humidity was constant at 50%, and nutrient solution was replenished on day 6. At 8 d seedlings were treated with either ABA ((±)-*cis*, *trans*-abscisic acid, Sigma, St. Louis, MO), NaCl or PEG (MW 8000, Sigma) by dissolving the appropriate volume of stock solution into the nutrient solution to obtain a final concentration of 125 μM ABA, 150 mM NaCl or 20% PEG. Control plants were grown at the same time as the treated plants. Tissue was harvested at 3 and 27 h post-treatment from a pool of at least 10 plants per trial. Shoot and root tissue was quickly divided at the residual seed coat, flash frozen in liquid N_2 and stored at −80 °C. The timing of germination, seed transfer, feeding, treatments and collection were kept constant among experiments to reduce the impact of circadian variation.

Experimental replicates are temporally distinct experiments, while biological replicates represent independent, spatially separated hydroponic vessels in which seedlings were grown at the same time within the same growth chamber. The overall experimental design used in this study is shown in Figure 2. The biological replicates of control and treated plants were obtained from one large experiment (PEG) or from two experiments done at different times (NaCl, ABA) (Figure 2). Technical replicates consist of microarray assays that were replicated in order to assess variability due to technical aspects of probe generation, slide to slide variation, and data acquisition. A total of seven to twelve replicate assays were obtained and evaluated for each treatment, time, and tissue combination (Table 2).

Generation of sorghum *microarrays*

cDNAs representing unique gene clusters (Pratt, *et al*., 2005) were amplified in 150 μl PCR reactions with modified T3 and T7 primers: Qfor7-CGACGGCCAGTGAATTGTAATACG, Qrev7-GGTGGCGGCCGCTCTAGAACTA. For 3′ EST clusters with two or more members, the cDNA clone chosen to represent that cluster was at least 99% identical to the consensus sequence and had the longest 3′ UTR. Reactions were purified using Montage PCR filter plates (Millipore, Billerica, MA), dried and resuspended in 1× Micro Spotting Solution to a final concentration of ~250 ng/μl. Of the 12 982 successfully amplified sequences confirmed by gel electrophoresis, the numbers of ESTs from each library were as follows: dark-grown (961), drought-stress after flowering (692), drought-stress before flowering (566), embryo (1041), floral meristem (1035), immature panicles (1284), light-grown (1541), 2 ovary libraries (1089), pathogen-induced (826), pathogen-infected (1496), 2 rhizome libraries (1138), and a water-stress library (1313). Additionally, >768 clones were confirmed to be accurate by resequencing. PCR products were arrayed onto UltraGAPS slides (Corning, Corning, NY) using an Omnigrid 100 microarrayer (Genomic Solutions, Ann Arbor, MI). Arrays were U.V. cross-linked (600 mJ), and processed with agitation as follows before use: 10 m in 0.6% SDS at room temperature, 2 m in boiling water, and 2 m in 95% ethanol at −20 °C.

Microarray analysis

Total RNA was isolated from root and shoot tissue separately using Trizol reagent with the suggested modification for plants (Molecular Research Center, Cincinnati, OH). First strand cDNA was made by reverse transcribing 40 μg of RNA using Superscript II (Invitrogen, Carlsbad, CA) with RT polyA-capture primers as described for the Array 350RP kit (Genisphere, Hatfield, PA). A two-step hybridization protocol similar to that described by Genisphere was performed with the following modifications. cDNA equivalent to 8 μg of total RNA from both control and treated tissue were hybridized together in the 1× SDS buffer from Genisphere at 65° C overnight under a lifter slip (Erie Scientific, Portsmouth, NH) in a sealed humid hybridization cassette (Monterey Industries, Richmond, CA). Secondary hybridizations with Cy3 and Cy5 dendrimers were performed essentially as the primary hybridization in 1× SDS buffer at 65° C for 2 h and washes were performed as suggested by the manufacturer. Photobleaching and fading of the fluorescent dyes was prevented by adding DTT to the wash buffers at a final concentration of 1 mM, protecting samples from light exposure, and coating slides with DyeSaver Antifade coating (Genisphere) prior to scanning. Slides were scanned using a ScanArray 5000 (Packard Bioscience, Billerica, MA) to generate balanced Cy3 and Cy5 images.

Data analysis

Images were processed using MolecularWare (MolecularWare, Inc., Cambridge, MA) and data was analyzed using Microsoft Excel and Spotfire (Spotfire, Inc., Sommerville, MA). All data has been deposited in a MIAME-compliant database (Brazma *et al.*, 2001) at http://fungen.org/Sorghum.htm (Cordonnier-Pratt *et al.*, 2004b). For the initial self vs. self-hybridization, raw data (unnormalized, unsubtracted) was used to assess any dye bias. Control vs. control hybridizations to assess biological and technical variability were background subtracted and normalized via a Lowess approach (Quackenbush, 2002) prior to sub-analysis at different replicative depths. Control vs. treatment hybridizations were background subtracted, normalized, and Cy3/Cy5 ratios were log-transformed prior to multiple slide analysis. Statistical analysis employing a Thompson test was used to identify and exclude outlier data points from ratio averages.

BLAST analysis

Sorghum EST sequences were initially subjected to BLAST analysis against the PIR database with no stringency cutoff, as well as to the nr peptide database downloaded from GenBank (May, 2004) at a cutoff of e−04. To determine the species distribution of the osmotically inducible unknown genes, ESTs were queried against both the nr and EST databases with an e-value cutoff of e−04. If the top hit was uninformative (i.e. gene of unknown function) the next best hit with a known function was frequently used. Most nr and EST BLAST results can be found in a database at http://sorgblast2.tamu.edu/EstSearch.html. Additional BLAST data obtained against the PIR-NREF database, filtered to exclude protein sequences judged not to be full length, as well as an interface for exploring the 3′ EST clusters that served as the basis for construction of this microarray, can be found at http://fungen.org/Sorghum.htm (M.M. Cordonnier-Pratt, personal communication).

qRT-PCR analysis

cDNA was made from total RNA obtained from equal amounts of at least three independent biological replicates per sample using random hexamers and TAQMAN reverse transcription reagents (Applied Biosystems, Branchburg, NJ). Quantitative real time PCR was performed on an Applied Biosystems 7900HT machine using SYBR chemistry for candidate genes identified by microarray analysis. Primers and probes were designed using Primer Express software (Applied Biosystems) to allow for amplification of ~100-bp products of similar GC and Tm characteristics. The generation of specific PCR products was confirmed both by melting curve and gel analysis. All primer sequences can be found in supplemental table III.

Thermal cycling conditions were 2 m at 50 °C and 10 m at 95 °C followed by 47 cycles at 95 °C for 15 s and 60 °C for 1 m. Samples were performed in duplicate and data was analyzed using the ABI PRISM 7900HT SDS software (Applied

Biosystems). Quantification was achieved using the comparative CT method (Bieche *et al.*, 1999), which consists of normalizing the number of target gene copies to an endogenous reference gene (18S rRNA, detected using the ribosomal TAQMAN kit supplied by Applied Biosystems). Fold inductions were calculated as $2\hat{}(\Delta CT_{control} - \Delta CT_{treatment})$.

Variability of RT-PCR results among biological replicates was determined by analyzing the mRNA levels of eight genes in RNA derived from three biological replicates from three different treatments (data not shown). The standard deviation among replicates ranged from 0.01 to 1.39 ΔCT with an average standard deviation of 0.82 ΔCT among replicates. Technical variability on assays of the same RNA sample was determined by performing duplicate assays on all genes (data not shown). An average error of 0.16 ΔCT was obtained for the entire data set, with less than 1.4% of the genes assayed having a greater error than 1 ΔCT (supplemental table II – highlighted genes). Therefore, on average, qRT-PCR values fall within a ±1 ΔCT range of the reported value corresponding to a confidence interval of ±2-fold variation.

Results

Sorghum *microarray development and assessment*

In a collaborative sorghum genomics project 117 682 ESTs, both 3′ and 5′, were sequenced from 14 cDNA libraries derived from different tissues, developmental stages and plants subjected to abiotic and biotic stresses (http://fungen.org/Sorghum.htm). The 3′ ESTs were clustered into 16 801 contigs, referred to as Milestone Ver. 1 (Pratt, *et al.*, 2005). 12 982 cDNAs were successfully amplified by PCR as judged by agarose gel electrophoresis from the 16 801 sequence contigs (data not shown). While all 16 801 PCR attempts were spotted, these 12 982 were the focus of this investigation. Microarrays and hybridization conditions were optimized and their results evaluated by performing self vs. self-hybridizations in order to assess inherent noise in the assay and dye bias resulting from dendrimer labeling. The raw mean Cy3 and Cy5 intensities plotted in Figure 1 reveal an approximate equality in hybridization in all spots indicating that there is limited confounding dye bias. Furthermore, 98% of the data fall within (±) 2-fold of an equal Cy3/Cy5 ratio. RNA corresponding to 7698 genes (59%) was reliably detected with a signal to background ratio >3-fold when control plants from nine experiments were analyzed. Since various growth perturbations can increase the expression of low abundance transcripts, data from all spots on the array were collected and subsequently filtered as necessary.

Variability of microarray results was assessed by examining results obtained with control plants grown under identical conditions but at different times and from different regions in a growth chamber. This experiment revealed that on average, signals from 5% of the spots on the array varied >2-fold when RNA samples from two different control samples were compared (Table 1). To determine the level of replication needed to minimize background variability, comparisons between control plants from four independent samples were analyzed at different depths of replication (2–9 slides/analysis). Table 1 lists the number of genes with variation exceeding thresholds from 1.8- to 10-fold for each level of replication. As expected, with increasing replication of control vs. control RNA, the average variation in signals from any given spot decreased. For example, analysis of 6 slides results in the detection of only ~9 genes (0.07%) having signal

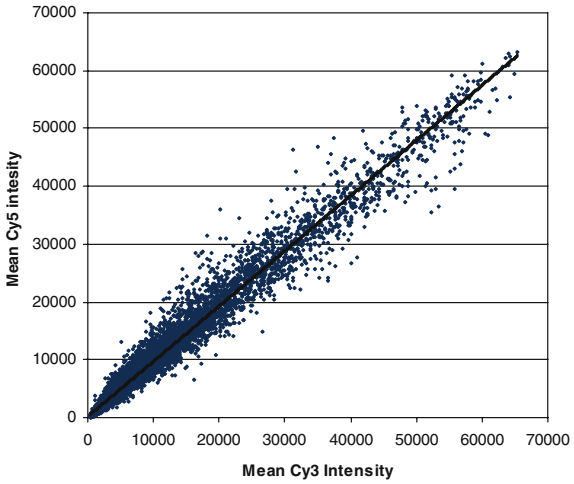

Figure 1. Self vs. self-hybridization. The same RNA was differentially labeled with Cy3 and Cy5 and hybridized to determine variance, dye bias and spot intensity range. Raw mean intensity for Cy5 is plotted against raw mean intensity for Cy3.

Table 1. The influence of replication on the discovery of false positive gene expression at different thresholds in control vs. control microarray hybridizations.

Replicative depth	Possible combinations	Fold change in gene expression				
		10	5	3	2	1.8
1	9	3.0 (0.02%)[a]	23.8 (0.18%)	118.2 (0.91%)	646.0 (4.98%)	1099.9 (8.47%)
2	36	0.0 (0.00%)	1.9 (0.01%)	15.6 (0.12%)	152.9 (1.18%)	298.1 (2.30%)
3	84	0.0 (0.00%)	0.0 (0.00%)	4.0 (0.03%)	64.2 (0.49%)	123.3 (0.95%)
4	126	0.0 (0.00%)	0.0 (0.00%)	1.4 (0.01%)	31.7 (0.24%)	72.1 (0.56%)
5	126	0.0 (0.00%)	0.0 (0.00%)	0.3 (0.00%)	17.1 (0.13%)	39.3 (0.30%)
6	84	0.0 (0.00%)	0.0 (0.00%)	0.0 (0.00%)	9.3 (0.07%)	25.8 (0.20%)
7	36	0.0 (0.00%)	0.0 (0.00%)	0.0 (0.00%)	4.7 (0.04%)	15.3 (0.12%)
8	9	0.0 (0.00%)	0.0 (0.00%)	0.0 (0.00%)	1.9 (0.01%)	9.7 (0.07%)
9	1	0.0 (0.00%)	0.0 (0.00%)	0.0 (0.00%)	2.0 (0.02%)	4.0 (0.03%)

[a] Average number of genes showing a change in expression and the % change.

variation greater than 2-fold in control vs. control experiments. After analyzing eight hybridization replicates, ~10 genes (0.07%) would be incorrectly thought to be differentially regulated >1.8-fold in a control vs. control experiment. Therefore, to reduce biological and technical variability of microarray analyses and to increase the statistical significance of the results, at least seven replicates per condition derived from at least four independent biological experiments were utilized.

ABA, salt and polyethylene glycol treatments

One goal of this study was to identify a broad spectrum of sorghum genes that are modulated in response to ABA, salinity and osmotic stress. In addition, we were interested in comparing changes in sorghum gene expression to those reported for Arabidopsis (Hoth *et al.*, 2002; Kreps *et al.*, 2002; Seki *et al.*, 2002b), rice (Kawasaki *et al.*, 2001; Rabbani *et al.*, 2003), barley (Ozturk *et al.*, 2002), and maize (Wang *et al.*, 2003). These preceding studies relied primarily on exposure of plants to relatively severe stress treatments followed by analysis of changes in gene expression over a period of hours or days. To permit comparison of the present results with studies in other species, sorghum seedlings were treated with ABA (125 μM), NaCl (150 mM), and polyethylene glycol (PEG; 20%), treatments that have been reported to elicit a myriad of physiological responses in plants. For sorghum, 125 μM ABA

Table 2. Repetition of osmotic arrays per condition and summary of gene regulation.

# hybs	*10*	*9*	*12*	*10*	*12*	*11*	*10*	*11*	*8*	*8*	*11*	*7*
Treatment[a]	ABA3R	ABA27R	ABA3S	ABA27S	SALT3R	SALT27R	SALT3S	SALT27S	PEG3R	PEG27R	PEG3S	PEG27S
Induced												
>10[b]	17[c]	16	8	22	0	0	1	0	0	0	16	3
>5	63	64	34	63	2	0	4	5	0	0	50	25
>3	150	127	98	169	33	1	16	15	1	1	131	127
>2	286	265	325	372	189	73	90	57	49	9	280	428
>1.8	405	333	480	507	341	138	142	106	118	21	356	607
Repressed												
>10	0	0	0	1	0	0	0	0	0	0	0	0
>5	5	1	1	19	0	1	0	0	3	0	1	0
>3	47	14	10	110	0	7	0	0	13	1	20	23
>2	220	40	177	361	50	23	15	15	63	17	134	659
>1.8	354	79	309	501	119	54	64	49	114	39	247	992

[a] R = 3 h roots; 27R = 27 h roots; 3S = 3 h shoots; 27S = 27 h shoots.
[b] Fold change in expression.
[c] Number of genes showing a change in expression for each treatment.

was selected because similar concentrations have been used in gene expression studies in other species, and because increasing the concentration of ABA above 125 μM did not cause additional changes in gene expression (data not shown). Similarly, 20% PEG-8000 (water potential = −0.51 MPa) has been used previously to induce plant dehydration (Verslues *et al.*, 1998) and this treatment caused pronounced leaf wilting and inhibition of root and shoot growth. While the relative water content of PEG-treated sorghum seedlings decreased ~30% within 3 h of treatment and remained at that level for the 27 h treatment, plants could recover after the 27 h PEG treatment although some root death was observed (data not shown). PEG can create a low oxygen condition in hydroponic media even when aeration is utilized as in this study (Verslues *et al.*, 1998). However, this potential secondary effect appears to be limited in the current study because genes activated in response to low oxygen conditions, such as alcohol dehydrogenases (Dolferus *et al.*, 1994), were not induced significantly in the PEG-treated sorghum seedlings. Treatment of rice, barley, and maize seedlings with 150 mM NaCl has been used to analyze salt induced changes in gene expression (Kawasaki *et al.*, 2001; Ozturk *et al.*, 2002; Wang *et al.*, 2003). Thus, hydroponic growth solutions were supplemented with 150 mM NaCl, which caused some leaf chlorosis in sorghum, and growth inhibition that could be reversed if seedlings were returned to normal salt concentrations (data not shown).

Microarray analysis of sorghum treated with ABA, NaCl and PEG

Microarrays were hybridized with Cy3- and Cy5-dendrimer labeled cDNA probes generated from RNA extracted from treated and untreated plants. A maximum of 12 data sets for each tissue/time point combination was obtained for each treatment (Figure 2). Data from some slides failed to pass quality control and were thus eliminated from analysis, resulting in a range of 7–12 slides derived from at least four different biological replicates per treatment (Table 2). Average Cy3/Cy5 ratios (fold changes) in RNA levels obtained from all microarray analyses are presented in supplemental table I. The number of genes that responded to the different treatments with changes in mRNA abundance is summarized in Table 2. Overall, expression of a greater number of genes was induced in response to ABA or NaCl when compared

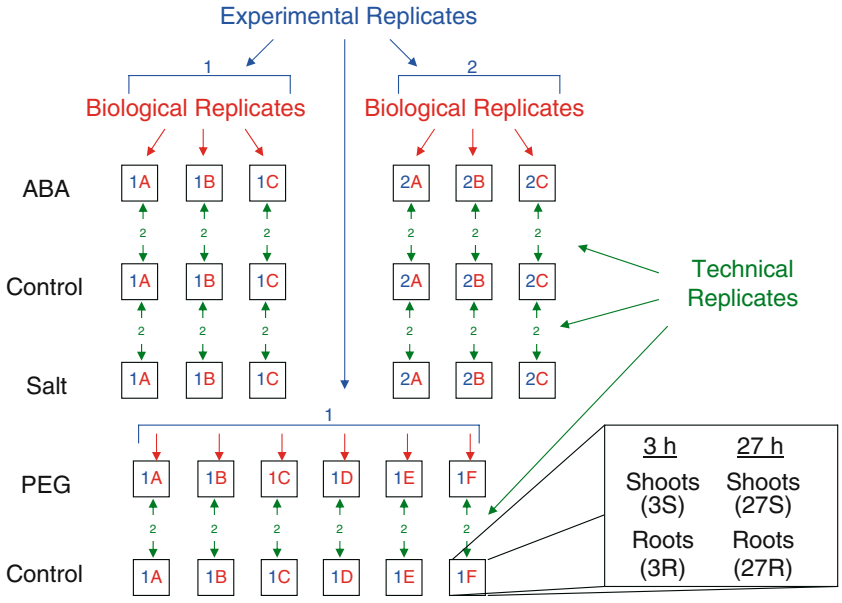

Figure 2. Experimental design and replication. The experimental design for the ABA, salt and PEG treatments is outlined, with blue numbers reflecting experimental replication and red letters indicating biological replication. At each comparison, a maximum of two technical replications was performed at the slide level. For each sample, tissue was collected at 3 and 27 h post-treatment and separated into root and shoot pools.

with the numbers of repressed genes, while PEG treatment repressed the expression of a greater number of genes when compared to the number of genes whose expression was enhanced. ABA treatment caused the largest relative changes in mRNA abundance observed with microarrays with 22 genes exhibiting >10-fold induction in shoots at the 27 h time point. Among the different treatments, PEG modulated the largest number of genes with 992 genes repressed >1.8-fold and 607 genes induced >1.8-fold in shoot tissues at the 27 h time point. In contrasting the relative effect of the three stresses on shoot vs. root tissues, high salt modulated the expression of a greater number of genes in root tissues, while the opposite was true for plants treated with PEG or ABA. In total, the expression of 1372 (10.5%), 583 (4.5%) and 1347 (10.4%) genes was modulated at least 2-fold in one or more tissue/time point combinations in response to ABA, NaCl, and PEG treatment, respectively.

Genes modulated by ABA, NaCl and PEG and their predicted functions

BLASTX analysis of genes modulated by ABA, NaCl and PEG treatments identified numerous sorghum genes predicted to belong to pathways that function in osmotic stress responses as well a number of ESTs of unknown function (Figure 3, supplemental table I). The genes induced >2-fold on microarrays were initially classified according to their involvement in regulation and signaling, growth, transport, metabolism, role in protecting plants from abiotic or biotic stress, and genes with unknown function (Figure 3). Overall ~68% of the genes modulated by ABA, NaCl or PEG corresponded to previously characterized genes with known or presumed functions. In addition, 174 genes modulated by one or more of these treatments did not return informative BLASTX results from the non-redundant (nr) database and were classified as unknowns. To obtain further information about these genes, the corresponding sorghum ESTs were analyzed using BLASTN to the EST, high throughput genomic sequence, and nr databases. Twenty-three genes (13%) had homologs in evolutionary lineages ranging from bacteria to higher eukaryotes, while the remaining 151 (87%) genes were restricted to plant species (Figure 3). Of these, 62 were found in both monocot and dicot plants, while 89 were found exclusively in monocot lineages. Interestingly, not all of the sorghum monocot-specific genes were found in rice at a cut off of e−04, even though a nearly complete rice genome sequence is available (Goff *et al*., 2002; Yu *et al*., 2002). Rice homologs could not be found for 29 unknown sorghum

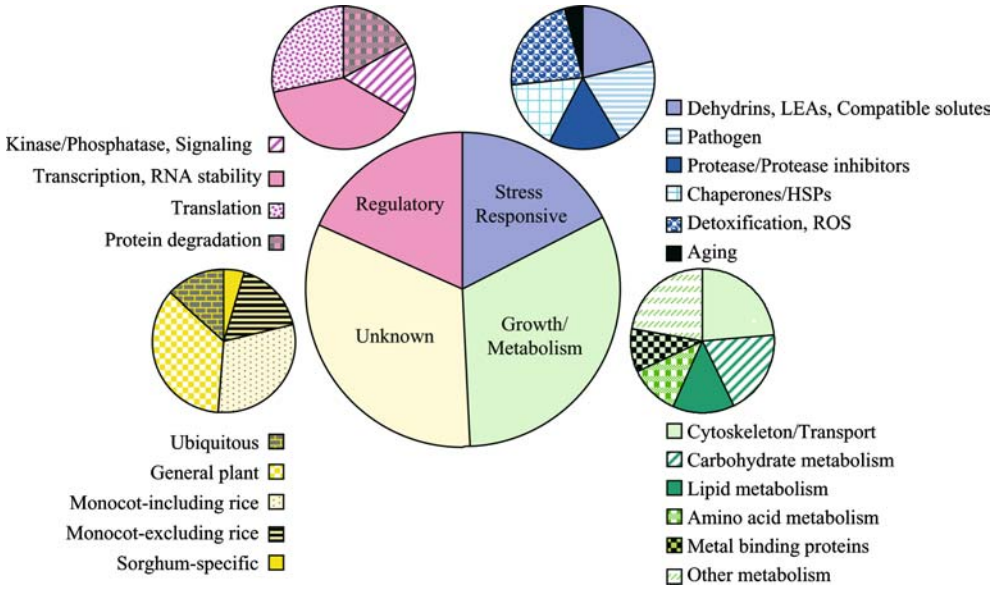

Figure 3. Functional classification of osmotically induced genes. The function of the 536 genes induced >2-fold by ABA, PEG or salt as determined by microarray analysis were classified into functional categories following BLASTX analysis.

genes although homologs could sometimes be found in species more closely related to sorghum than rice such as sugarcane and maize. Finally, homologs of 8 sorghum ESTs were not identified using a cut off of e−04 and these ESTs were thus classified tentatively as 'sorghum-specific' (CF757441, CF772738, BI141499, AI724771, BG159069, AW745667, AW680488, and BE592280). However, several of these EST sequences were relatively short (107–125 bp), therefore, further sequence analysis may reveal homology to known genes.

Many of the 538 genes regulated in response to treatment with ABA, PEG and/or salt function in growth related processes and cell wall biosynthesis consistent with rapid perturbation of growth by osmotic stress and ABA. Expression of genes involved in amino acid, ion, glucose and water transport and genes encoding enzymes involved in metabolism, photosynthesis and respiration was also modified by ABA, NaCl or osmotic stress. As expected, a large number of genes induced by NaCl, PEG and/or ABA encode proteins that protect plants from abiotic and biotic stress. In addition, a diverse group of genes that protect cells from reactive oxygen species and ~21 genes involved in detoxification were modulated by at least one of the three stress treatments (supplemental table I). Additionally, 20 genes modulated by ABA, NaCl or PEG treatment were previously reported to be regulated by hormones involved in plant defense (salicylic acid (SA), JA, or ACC/ethylene) (supplemental table I). Approximately 27% of the known genes modulated by ABA, NaCl and PEG treatments encode proteins predicted to have regulatory functions involved in signaling, transcription, RNA turnover/binding, translation, protein folding and protein turnover (supplemental table I). Furthermore, 30 genes encoding transcription factors and proteins involved in DNA methylation, chromatin structure and polyamine biosynthesis were regulated in response to ABA, NaCl and/or PEG (supplemental table I).

Tissue, kinetic and treatment specificity of gene expression

Microarray analysis revealed that the response of genes to ABA, high salinity, and cellular dehydration is complex and varies depending on the specific treatment, tissue, and the time point examined. For instance, of the 329 genes induced >3-fold by ABA, ~79% were induced at this level either in shoots or roots but not both tissues (Figure 4). Overall, ~65% of the ABA modulated genes were induced >3-fold at only one time point in one tissue and only 30 of the 329 modulated genes were induced in both tissues and time points following ABA treatment (Figure 4). This latter set of genes includes six dehydrins, two LEA proteins, one transcription factor and 12 genes of unknown function, among others. Additionally, most of the modulated genes were differentially responsive to ABA, NaCl or PEG treatment.

The overlap among genes induced >2-fold following treatment of plants with ABA, NaCl or PEG was analyzed separately for roots and shoots (Figure 5). This analysis showed that a large portion of the modulated genes were differentially responsive to ABA, NaCl or PEG treatments. In roots, 460 of the 553 genes induced >2-fold by these treatments (83%) were induced to this level in response to only a single treatment: PEG (36 genes), NaCl (130 genes) or ABA (294 genes) (Figure 5B). There was minimal overlap between PEG and NaCl responsive genes except for genes that were also responsive to ABA. In shoots 324 genes (43%) were induced by two or more of the treatments indicating greater overlap in gene expression response in this tissue to the three treatments. The greatest overlap of gene expression was observed between genes induced in shoots by PEG and ABA (Figure 5A).

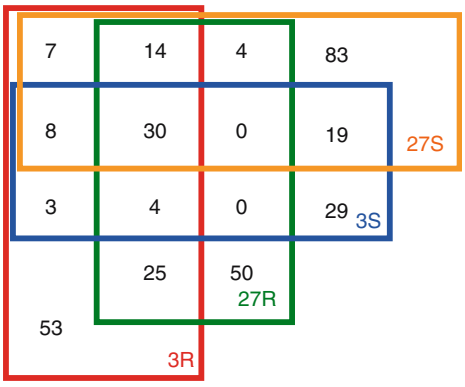

Figure 4. Tissue and time point specificity of gene expression in response to ABA treatment. This Venn diagram shows the expression pattern and overlap of 329 genes induced >3-fold by ABA as determined by microarray analysis for 3-h roots (3R), 27-h roots (27R), 3-h shoots (3S) and 27-h shoots (27S).

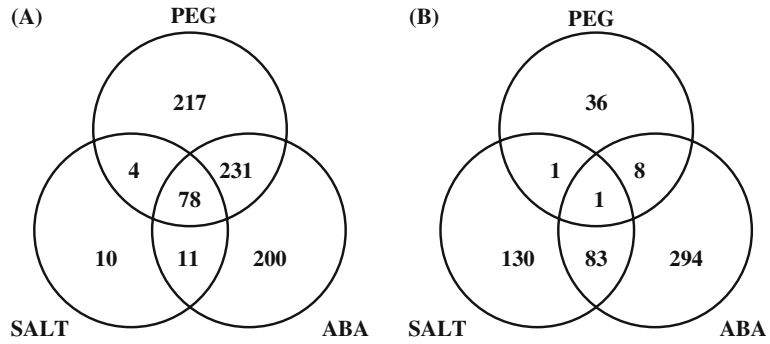

Figure 5. Treatment specificity and overlap of genes induced by ABA, PEG and salt in roots and shoots. Venn diagrams show the overlap among genes induced >2-fold in the shoots (panel A) or roots (panel B) at either the early or late time point by each treatment as determined by microarray analysis.

The regulation and potential function of specific sorghum genes that show altered RNA levels in shoots in response to seedling dehydration or ABA treatment was further analyzed using qRT-PCR because this technique has excellent sensitivity, specificity and dynamic range. The level of replication required to ensure statistical significance of qRT-PCR results was determined by analyzing technical variability of the qRT-PCR assay and variability among RNA samples obtained from the different biological replicates used for microarray analysis (see Materials and methods). This information allowed us to assay the expression of more than 300 genes using qRT-PCR with an average reported error of less than ± 2-fold at reasonable cost. The qRT-PCR assays detected up to several thousand-fold changes in the abundance of specific transcripts, a much greater dynamic range compared to microarray analysis. The difference in apparent fold-change in RNA abundance detected by the two methods may be due to cross-hybridization of gene family members on cDNA arrays, differences in primer efficiencies, and differences in the detection limits of the two methods. Overall, 256 genes identified through microarray analysis that showed relatively large changes in mRNA abundance and genes with variation in kinetic- or treatment-specific expression were selected for qRT-PCR analysis. Additionally, 77 other genes, including homologs of known stress-inducible genes, genes from a recently constructed cDNA library from NaCl-treated sorghum, and genes identified by screening the collection of methyl filtered genome sequences (http://www.plantgenomics.iastate.edu/maize/) were incorporated into the analysis. The results of the >10 600 qRT-PCR assays are reported in supplemental table II. A subset of the data on sorghum genes with homology to genes with known or predicted functions is shown in Table 3. These include osmotically regulated genes with functions in growth, transport or metabolism, protection and regulation (see below).

Discussion

Microarray, qRT-PCR analysis and experimental design

We have developed a cDNA microarray for the drought tolerant C4 monocot *Sorghum bicolor* that contains PCR products derived from 12 982 different gene clusters. This collection of gene sequences is expected to represent ~25% of the sorghum gene complement based on the prediction of ~50 000 genes in diploid grass genomes such as rice and sorghum (Goff *et al*., 2002). RNA was collected from six biological replicates of each experimental treatment and analyzed on duplicate microarray slides allowing data from a minimum of seven replicates to be combined for analysis (Figure 2). The experimental design minimized false positives due to biological and technical variation resulting in a high level of statistical significance for the data reported here. Microarray analysis was performed on RNA extracted from roots and shoots of 8-day-old seedlings treated for

Table 3. The effect of salt, PEG, and ABA on gene expression for a subset of genes analyzed by qRT-PCR. Genes are organized by functional category.

			Roots						Shoots							
			Salt		PEG		ABA		Salt		PEG		ABA			
GROWTH/TRANSPORT/METABOLISM		S/R[a]	3 h[b,c]	27 h	3 h	27 h	3 h	27 h	3 h	27 h	3 h	27 h	3 h	27 h	e-value	BLASTX identity
Growth/cell walls/cytoskeleton																
AW284460[d]	LG1_284_E08[e]	161.2	-5.7	-5.4	-170.8	-15.3	-2.6	-17.4	-2.0	-6.7	-17.6	-13.7	-3.4	-27.8	e-11	ZmGR1b protein
BE363707	WS1_69_B10	1.8	241.0	3.8	114.4	20.3	2292.7	2587.8	20.4	1.2	143.8	7.5	27.9	3.9	e-54	Actin depolymerization factor-like protein
AW285731	LG1_320_G12	1.3	3.6	3.9	-1.4	24.8	14.8	4.3	1.4	1.7	-1.6	1.9	2.1	2.3	e-16	Actin
AW282977	LG1_206_C12	-1.6	-2.5	-1.2	-3.8	-2.2	-9.3	-6.3	-1.1	-1.3	-3.5	-1.0	-2.6	-3.9	e-65	Actin
BI098997	IP1_36_A11	-2.4	-2.2	-2.6	-6.4	-4.0	-2.1	-1.9	-1.3	-1.8	1.3	-1.1	-1.0	-1.9	e-63	DWARF8
BG051261	FM1_54_C04	-40.9	1.9	1.8	-1.6	1.3	-2.0	1.5	257.7	179.2	104.3	81.4	106.5	33.6	e-06	Beta-expansin/allergen protein
Transport																
BE366434	PI1_33_A10	1194.0	6.8	15.6	1.5	3.7	16.5	31.1	2.9	1.7	1.1	6.4	2.6	8.2	e-41	Plasmalemma aquaporin (PIP2)
BM326785	PIC1_2_G05	50.0	-1.1	-2.6	1.8	1.6	2.0	2.1	-6.1	-46.7	-19.7	-26.9	-10.1	-281.2	e-45	Pyrophosphate energized vacuolar membrane proton pump
AW286943	LG1_262_D09	39.5	1.3	3.4	2.1	8.4	11.1	28.7	1.3	1.7	-1.4	1.0	-3.2	-3.5	e-16	Tonoplast membrane integral protein ZmTIP4-1
CF757790	DSAF1_22_C12	9.8	5.2	5.2	3.4	10.2	1.4	5.8	1.4	1.4	-5.7	2.0	1.1	2.8	e-05	ABC transporter-like protein
CF771652	DSBF1_22_D05	-1.1	-1.6	-2.1	-2.0	2.2	-1.2	1.9	3.3	2.2	6.4	3.0	2.2	2.2	e-25	Major facilitator superfamily antiporter
AW747287	WS1_67_B08	-1.6	-1.6	2.3	-1.5	-1.3	1.4	2.0	2.8	2.5	1.7	6.1	1.6	6.0	e-22	Pyrophosphate-energized vacuolar membrane proton pump
BG488182	RHIZ2_60_E08	-1.7	-5.8	-4.1	1.8	2.2	-3.0	-7.5	1.1	-1.4	1.0	3.3	-1.2	2.6	e-76	Delta-type tonoplast intrinsic protein
BG357473	OV2_30_C12	-7.2	-3.1	-3.4	-3.1	-1.7	-1.2	-2.1	-1.5	1.1	1.9	1.0	-1.4	-1.3	e-74	Glucose transporter
Photosynthesis																
BM317609	LG1_260_B02	1267.7	1.2	5.8	5.6	-7.8	6.9	1.5	-1.5	-1.7	-1.7	-1.4	-1.3	-3.8	e-76	RuBisCO small subunit
BM331226	PIC1_71_C11	606.3	1.4	3.8	-3.6	-8.1	1.9	2.3	1.1	1.2	-3.0	-1.8	-1.7	-3.1	e-36	Triose phosphate/phosphate translocator, chloroplast precursor
BM317714	LG1_272_D09	310.0	-1.3	1.2	-40.4	-12.3	1.2	-4.2	1.1	-1.2	-2.2	-1.6	-2.0	-4.7	e-50	Chlorophyll a/b-binding protein CP26 precursor
C4-photosynthesis																
BE596962	PI1_60_F07	1.1	3.6	1.9	1.3	-9.1	1.0	1.3	2.6	7.8	2.1	2.0	-1.3	2.2	e-98	Phosphoenolpyruvate carboxylase 1 (EC 4.1.1.31) (PEPCase 1) (CP21)
BG049597	EM1_6_C12	1.1	1.3	1.4	1.3	-20.4	-1.2	2.4	1.9	2.9	4.3	2.0	2.0	2.8	e-33	Phosphoenolpyruvate carboxylase 2 (EC 4.1.1.31) (PEPCase 2) (CP28)
BM329868	PIC1_44_C06	300.6	-1.6	1.6	-5.3	1.7	1.5	1.6	-1.3	-1.1	-1.4	1.3	-1.2	-1.6	e-54	Pyruvate orthophosphate dikinase
AW671792	LG1_351_A07	263.5	-1.1	1.8	-1.8	-2.2	1.2	2.1	1.6	-1.5	-1.9	-2.9	-2.2	-4.2	e-58	Malate dehydrogenase [NADP] 1, chloroplast precursor
BG560421	RHIZ2_74_B10	9.9	1.5	1.5	1.4	1.4	1.8	1.5	-1.2	1.6	-1.6	1.3	-1.0	-1.1	e-79	NADP-dependent malic enzyme
AW746273	WS1_40_E02	-2.5	87.4	2.9	14.8	7.3	8967.9	7656.5	77.4	14.1	859.6	57.0	353.5	432.8	e-49	NADP dependent malic enzyme
Carbon/Nitrogen Metabolism																
BF657337	OV2_20_F06	127.7	1.0	2.8	-1.0	1.5	2.2	4.2	1.8	2.6	-1.7	-1.2	-2.0	-3.2	e-57	Granule-bound starch synthase GBSSII
AW671747	LG1_351_F05	48.7	1.9	1.2	1.3	10.3	35.9	2.2	1.7	-1.4	-1.7	-2.3	1.7	-1.2	e-85	Pyruvate dehydrogenase kinase isoform 2
BG355487	EM1_17_B01	4.2	-2.7	-3.6	1.4	1.3	1.4	3.0	4.5	5.3	1.8	6.5	5.1	7.8	e-87	Glucose-1-phosphate adenylyltransferase large subunit 2
BM329727	PIC1_42_D10	-1.0	-1.7	-1.5	3.3	5.2	6.2	1.4	1.5	1.4	4.9	3.5	3.6	2.0	e-60	UDP-glucose 4-epimerase
AW747111	WS1_65_F03	-1.4	3.9	1.6	1.8	-1.3	20.6	69.1	6.4	6.3	8.5	18.1	3.6	12.6	e-15	Sucrose synthase 3
BI075957	IP1_23_E05	-2.5	-2.2	-2.7	-3.0	-2.1	-2.6	-1.8	1.5	-1.1	-1.1	-1.7	-1.7	-3.5	e-84	Glutamine synthetase
AW922489	DG1_19_B09	-4.9	1.0	1.6	1.5	1.9	-7.7	-1.9	-1.1	-1.6	-1.6	1.0	2.2	-1.3	e-48	Sucrose synthase 1
AW923055	DG1_48_G01	-14.7	-1.8	-1.6	-10.7	-2.6	-5.4	-3.6	1.4	-1.5	-1.0	-4.7	2.7	-1.7	e-58	O-methyltransferase ZRP4
BE367569	PI1_9_G03	-14.2	-3.0	-3.2	-3.2	-10.5	-3.7	-1.7	1.2	-1.5	2.6	1.2	1.6	-2.3	e-56	Sugar-starvation induced protein
Lipid metabolism																
AW286795	LG1_206_E08	396.5	8.0	6.1	41.7	13.1	2.5	-1.4	1.3	-1.1	-13.8	-8.1	-8.0	-13.8	e-68	Omega-3 fatty acid desaturase FAD7
BE366887	PI1_41_E11	219.4	-1.1	-1.4	-13.7	-2.4	1.4	1.7	-1.3	-1.0	-4.6	-1.1	-2.4	-2.5	e-08	Lipoxygenase 2
BG102888	RHIZ2_34_F05	5.7	1.3	1.0	1.0	1.4	1.9	1.3	-1.1	-1.3	-3.2	-3.1	-4.2	-7.5	e-26	delta-12 oleate desaturase
BE599325	PI1_88_B04	1.6	4.8	1.6	2.3	1.4	3.1	4.4	3.4	2.4	3.2	1.2	1.3	1.4	e-37	Choline kinase[imported]
BI140020	IP1_48_H07	-2.4	3.0	2.8	-2.4	1.4	-1.5	1.0	1.1	1.2	-3.9	-3.7	-1.3	-5.8	e-90	Choline kinase
BF480926	FM1_15_B03	-1.0	6.7	1.7	32.8	10.2	46.9	23.1	-1.1	1.1	5.1	-2.1	2.7	-1.8	e-16	Lipase
AW680781	WS1_7_D03	-2.0	16.0	4.9	10.4	-1.1	56.3	21.1	4.9	1.7	1.7	2.9	2.7	6.5	e-40	Epoxide hydrolase
AW283834	LG1_260_C09	-13.5	-2.1	-1.4	-5.6	-3.7	-2.9	-1.9	2.2	-1.2	3.8	-1.0	2.7	-1.1	e-34	Lipoxygenase
Nucleotides																
AW679534	WS1_29_G02	20.7	10.4	13.1	8.1	16.3	21.3	19.9	-1.3	2.7	-1.6	-47.8	-3.9	-58.2	e-24	Cytidine deaminase
AW744890	LG1_384_H09	-3.5	1.1	1.5	1.1	-1.5	2.1	-2.1	1.6	2.4	2.0	-1.6	11.6	2.5	e-21	CTP synthase
Metal binding proteins																
AW746842	WS1_55_D02	7.1	1.3	7.6	2.3	1.3	1.8	7.2	1.7	1.1	-1.3	1.1	-2.2	-1.6	e-29	Ferritin
BE357974	DG1_23_H02	4.1	-1.5	1.1	-1.5	32.8	10.4	1.7	1.2	-1.6	-1.3	1.2	1.3	1.1	e-06	Metallothionein
BF481787	FM1_20_D01	2.2	-1.9	-1.2	-5.4	-3.1	-13.3	-1.5	1.3	-1.0	-1.1	-1.7	-1.5	-1.2	e-49	Zinc-binding protein
PROTECTION																
Compatible solutes																
BG649294	EM1_80_F03	2.5	7.0	2.7	3.2	1.7	32.6	6.1	5.2	1.9	22.4	3.6	20.3	7.6	e-52	Inositol-3-phosphate synthase
CF757720	DSAF1_21_D10	1.3	1.7	3.7	2.1	-1.3	1.4	5.8	3.7	2.0	2.3	8.4	1.9	8.3	e-07	Delta 1-pyrroline-5-carboxylate synthetase (P5CS)
BI075477	IP1_21_A06	-1.9	4.8	2.2	-1.1	16.4	14.9	2.0	2.4	1.9	3.0	1.2	3.4	2.1	e-70	Aldehyde dehydrogenase
BF657321	OV2_20_D07	-5.5	-1.1	1.1	-1.1	-2.0	4.3	-2.4	1.1	1.5	-1.8	-1.1	1.5	1.1	e-22	Trehalose-6-phosphate phosphatase
BM318015	PI1_36_E09	-4.1	-2.0	-2.0	-2.9	-1.3	-4.4	-2.2	1.5	1.1	2.9	2.1	1.8	1.8	e-83	Choline monooxygenase
CD222415	CCC1_21_H07	-3.6	1.1	1.1	-1.7	1.1	-1.6	1.4	1.7	2.5	1.1	1.8	-1.2	1.4	e-90	Betaine aldehyde dehydrogenase
CF756052	DSAF1_3_E07	7.7	1.2	1.1	1.1	1.0	-5.9	-1.8	1.9	1.5	1.2	1.2	-1.2	-1.6	e-28	Trehalose 6 phosphate synthase

Table 3. (Continued).

			Roots						Shoots							
		S/R	Salt		PEG		ABA		Salt		PEG		ABA			
PROTECTION			3 h	27 h	3 h	27 h	3 h	27 h	3 h	27 h	3 h	27 h	3 h	27 h	e-value	BLASTX identity
Dehydrins/LEAs																
AW678291	WS1_14_D10	1.6	880.8	11.6	143.7	8.5	11413.5	28427.3	19.5	36.3	280.2	86.0	218.7	324.8	e-23	Late embryogenesis abundant protein, group 3 (LEA)
AW680922	WS1_8_F06	1.2	628.5	9.7	131.6	8.4	13221.5	17525.5	20.3	50.7	347.0	102.0	232.0	301.4	e-13	Late embryogenesis abundant protein, group 3 (LEA)
AW745922	WS1_38_F05	-1.2	564.2	8.8	301.3	52.4	14686.1	2133.4	3.2	94.8	1120.9	45.2	112.4	84.9	e-17	Dehydrin 6
AW679463	WS1_24_E04	-1.2	509.4	8.1	98.0	7.5	9793.9	14719.9	26.0	46.7	407.3	90.2	259.6	338.4	e-14	Late embryogenesis abundant protein, group 3 (LEA)
AW679264	WS1_23_H04	-1.2	6.8	4.0	8.0	10.5	1.2	4.4	1.1	13.5	-11.2	1.6	4.9	-7.9	e-10	Dehydrin 3
AW679430	WS1_24_G08	-1.2	406.5	5.5	84.7	6.2	6946.1	10320.9	17.4	25.1	262.3	72.3	158.4	227.7	e-15	Late embryogenesis abundant protein, group 3 (LEA)
CF771697	DSBF1_23_A06	-1.6	9.8	2.3	22.2	5.3	44.8	34.3	2.0	-1.9	8.4	1.7	2.0	-1.9	e-16	Dehydrin DHN2
AW679445	WS1_24_B11	-3.0	6.1	1.2	10.3	6.3	410.0	300.2	36.4	-1.8	218.0	53.3	84.2	346.2	e-22	Dehydrin DHN2
BE363366	WS1_62_B01	-12.1	3.5	-1.3	3.0	2.0	134.5	377.6	87.9	29.4	319.4	63.5	163.7	552.6	e-28	Dehydrin DHN2
AW678146	WS1_13_C11	-17.0	6.7	2.3	-1.4	-1.6	116.1	406.7	151.7	414.4	861.4	34.9	404.3	1788.7	e-10	Dehydrin DHN2
AW679462	WS1_24_E05	-17.5	4.0	-1.5	2.1	2.6	58.9	289.6	81.8	-2.1	344.6	152.2	208.9	96.9	e-17	Dehydrin DHN2
Other stress proteins																
AW747649	WS1_74_F10	9.4	25.2	2.8	11.3	1.3	269.3	128.3	24.2	2.8	29.3	1.2	32.2	9.8	e-15	Oleosin 2
AW746846	WS1_55_B05	-3.6	1.5	3.0	22.6	2.0	5.9	7.7	4.6	4.9	5.0	9.4	2.7	18.1	e-08	Dermal glycoprotein
CF758900	DSAF1_37_H08	-755.4	2.1	-1.4	nd	1.2	-1.5	-3.4	4524.8	1216.8	4864.4	2168.7	3100.1	2413.7	e-13	drought inducible 22 kD protein
ROS																
CF771397	DSBF1_18_G04	-1.1	-1.1	-1.0	-1.5	-4.9	-1.3	1.4	2.0	2.8	-1.0	11.0	1.7	13.3	e-52	Thylakoid-bound ascorbate peroxidase
BI245420	IP1_65_A09	-1.9	-3.5	1.7	1.1	-3.5	-1.2	1.5	2.0	1.3	1.7	7.0	2.0	4.5	e-34	Superoxide dismutase [Cu-Zn] 4A
BM329698	PIC1_42_H01	-2.5	3.0	-1.0	-2.0	1.3	1.0	4.0	2.1	2.2	1.6	1.2	-1.4	1.1	e-36	Catalase isozyme 1
Defense																
AW676997	DG1_3_D02	901.1	1674.7	1060.0	1832.1	1139.4	63.2	1542.9	1.6	2.1	1.0	2.0	1.5	1.1	e-48	Disease resistance-like protein
AW565363	LG1_343_C04	627.4	-2.1	-2.7	-16.6	-36.3	4.0	-5.8	-2.8	-4.9	-12.7	-9.9	-2.7	-16.8	e-23	Protease inhibitor
CF757359	DSAF1_17_A02	2.2	1.3	-1.5	2.9	4.9	2.6	4.6	3.1	1.7	3.6	2.2	3.6	4.4	e-57	Cysteine proteinase inhibitor
BG557573	EM1_53_C10	-2.7	-1.1	1.6	6.3	-1.6	3.5	2.5	-1.0	-1.2	1.2	-1.7	1.2	-3.2	e-57	Oryzain beta chain precursor
CF772835	DSBF1_40_A01	-4.4	-2.8	-3.1	-6.5	-2.7	-3.5	-1.1	2.2	1.4	1.2	1.7	-1.5	-1.1	e-17	NBS-LRR-like protein
AW283395	LG1_223_F07	-11.7	-13.4	-14459	6.5	10.8	8.6	5.5	1.3	4.1	4.4	14.9	1.5	16.1	e-17	Xylanase inhibitor protein
AW747623	WS1_74_C11	-11.7	2.4	2.4	2.8	4.4	13.1	13.6	2.1	10.5	7.6	35.2	274.9	43.4	e-23	Xylanase inhibitor protein
BE362384	DG1_86_G12	-13.0	-6.8	-8.8	-5.5	-2.8	-2.7	-4.2	-1.0	-1.2	1.4	1.2	-2.3	-3.5	e-25	NBS-LRR type resistance protein
AW679126	WS1_22_C05	-15.4	-1.1	-1.7	5.5	9.5	7.7	4.7	1.6	3.8	5.2	13.4	1.4	24.4	e-23	Xylanase inhibitor protein
CF771131	DSBF1_14_D03	-25.1	-1.0	-4.4	1.6	12.7	22.4	44.6	1.8	1.4	5.9	1.4	1.2	4.5	e-24	Subtilisin/chymotrypsin inhibitor
AW746284	WS1_40_H09	-27.7	-1.1	-2.6	-1.1	4.4	14.7	52.3	5.5	6.8	15.2	7.3	2.1	21.1	e-25	Subtilisin/chymotrypsin inhibitor
Other																
AW679048	WS1_22_G03	-10.8	6.9	2.3	-1.4	-2.0	100.9	394.5	117.6	58.4	595.9	319.0	257.1	790.8	e-21	Senescence-associated protein
BG240861	OV1_39_B09	8.5	-3.0	1.0	2.0	5.2	21.0	9.8	-4.8	-3.9	-3.6	-17.2	1.4	-1.0	e-46	Phi-1 protein
AW745824	WS1_37_H06	-2.5	-1.2	-1.2	-1.1	1.0	4.5	3.9	4.6	6.2	12.8	23.3	11.6	12.9	e-34	2-oxoglutarate-dependent dioxygenase
REGULATION																
Kinase/phosphatase																
CF758134	DSAF1_27_G12	5.6	-1.7	-3.3	-1.1	1.0	-1.5	-1.2	-1.3	-1.1	-1.9	-1.6	-3.6	-5.4	e-13	Protein kinase [imported]
CF772114	DSBF1_29_D06	5.6	13.8	3.8	12.6	4.0	13.8	30.9	-3.3	2.3	1.0	2.1	4.0	1.3	e-34	Serine/threonine protein phosphatase PP2A-3 catalytic subunit
BG102689	RHIZ2_36_A12	1.6	3.8	1.7	2.8	1.5	3.6	6.5	3.3	2.8	4.3	1.6	1.7	1.7	e-54	Serine/threonine Kinase
AW284269	LG1_268_H02	1.2	-4.9	-5.9	-2.1	-1.7	-4.2	-1.6	1.1	-1.4	-1.0	-1.2	-2.8	-2.9	e-26	Protein kinase
BM326663	PIC1_71_A03	1.0	1.6	-1.5	10.0	4.5	16.1	13.2	3.0	-1.2	5.3	1.6	6.3	1.8	e-35	Protein phosphatase 2C-like protein
Signaling - other																
BM328853	PIC1_27_D07	5.8	-2.0	-2.1	1.4	1.4	-3.4	1.3	1.3	-1.1	-2.4	-2.0	-2.9	-3.4	e-05	GTPase activating protein
BI245807	IP1_64_F08	1.8	1.3	1.2	5.7	3.0	1.0	2.3	-1.1	-1.8	-3.0	-3.7	-1.9	-2.6	e-07	Phosphoinositide kinase
AW672256	LG1_358_H12	-50.0	6.3	-3.4	-1.3	-1.1	-1.8	1.5	7.3	-5.2	2.4	-2.7	-1.1	-1.6	e-102	ADP-ribosylation factor
Transcription factors																
BG054221	OV2_3_G06	4.9	-13.1	-2.8	-5.9	-2.7	-71.8	-2.7	-1.1	1.0	-2.0	-2.3	-2.2	-1.9	e-57	MADS-box protein 9
BI098182	IP1_29_B06	1.8	2.7	18.9	2.2	60.5	20.7	19.6	1.7	6.1	6.2	8.5	13.0	15.0	e-37	NAM (no apical meristem) protein

Hormone biosynthesis															
SAMIv1.0_46876		292.8	1.8	18.3	-6.6	1.2	-2.2	4.8	1.5	1.4	-3.0	-1.3	-3.4	-3.4	e-116	9-cis-epoxycarotenoid dioxygenase
SAMIv1.0_54439		2.7	67.3	16.6	144.7	130.0	3198.4	611.8	6.8	2.6	34.9	4.8	7.0	4.1	e-82	9-cis-epoxycarotenoid dioxygenase
SAMIv.10_49229		-2.2	1.6	2.3	30.8	14.5	6.0	2.3	32.3	2.6	184.3	25.9	29.1	3.9	e-180	9-cis-epoxycarotenoid dioxygenase
Transcription factors																
BF586407	FM1_28_D08	1.8	1.5	-1.5	5.6	1.9	8.3	1.4	-2.0	-2.2	-6.5	-6.8	-4.2	-9.1	e-14	CONSTANS-like protein CO7
BG410796	EM1_26_A11	1.5	-1.4	-1.1	-1.3	-1.5	-1.5	1.7	1.4	-1.1	-2.7	-3.7	-4.3	-3.6	e-79	DNA-binding protein GBP16
CF769808	DSBF1_4_B04	1.2	-1.5	-2.9	1.0	-1.2	-1.0	-1.4	1.1	-2.0	-3.8	-1.4	-1.6	-1.5	e-50	CONSTANS-like B-box zinc finger protein
BE366742	PI1_3_E01	1.2	-1.4	-1.3	21.9	1.6	6.8	1.6	1.2	-1.3	2.4	-1.2	2.0	1.6	e-17	WRKY transcription factor
AW922845	DG1_46_C03	-1.4	-1.4	1.1	-1.3	-3.4	-1.6	-1.2	1.3	1.0	1.3	4.0	1.1	3.7	e-37	Zinc finger protein
BM326994	PIC1_5_G02	-1.5	-1.7	-1.4	2.0	1.3	4.0	10.0	2.3	1.1	4.3	2.1	1.5	1.2	e-13	G-box binding factor
BG556883	EM1_39_H01	-1.6	1.0	1.2	-4.6	1.1	-5.1	1.4	1.1	1.8	-1.4	2.1	-1.9	-1.8	e-31	EREBP-type transcription factor
BM324557	PIC1_32_G02	-1.7	1.4	1.1	8.2	15.4	20.9	20.4	7.1	1.6	18.4	10.7	17.9	21.4	e-28	Zinc finger transcription factor
BG463265	EM1_48_D11	-1.8	1.0	2.3	-1.0	-1.9	-1.4	2.2	2.8	2.0	1.4	5.2	1.2	4.8	e-54	WD-40 repeat protein-like
BE918289	OV1_1_E11	-2.2	-1.4	34.0	1.1	7.7	6.6	1.2	-1.5	1.5	-1.2	1.7	3.4	1.5	e-44	NAC4 protein
BE357696	DG1_21_D11	-2.4	-2.2	-1.8	-3.4	1.0	1.0	-2.1	-1.4	-1.5	-1.1	-1.3	-1.5	-1.1	e-20	SET-domain transcription regulator
BI098357	IP1_31_D05	-2.7	3.2	1.1	1.5	-1.7	8.7	2.6	1.5	1.8	2.0	2.4	-1.7	-1.3	e-28	AP2 domain transcription factor
CF756433	DSAF1_5_B08	-4.4	-2.3	-2.5	3.2	1.0	5.6	3.8	-1.3	-1.4	3.6	1.4	-1.1	-2.8	e-08	MADS box protein
BG241945	RHIZ2_17_F03	-8.3	-1.3	2.5	-1.0	-1.6	2.1	4.1	3.0	3.0	2.3	14.9	2.6	31.5	e-33	Myb-related transcription factor
AW676733	DG1_13_G11	-12.8	-2.2	-2.9	-2.7	3.4	4.2	-1.9	2.1	1.2	7.4	1.7	4.3	2.4	e-29	DNA binding protein
BE363303	WS1_61_A11	-16.7	-1.0	1.9	1.2	-1.9	2.1	2.1	10.4	4.4	8.6	9.7	10.5	25.2	e-51	HD-zip transcription factor
BE357073	DG1_146_C10	-17.0	8.0	-6.7	2.0	-1.4	-1.8	2.5	2.6	4.3	2.4	3.5	1.3	3.1	e-20	Transcription factor Myb4
DNA-structure																
BE917633	OV1_6_B07	6.7	-1.0	1.0	1.3	1.9	-2.7	1.4	1.1	-1.5	-4.2	-6.4	-4.2	-12.8	e-39	histone H4 homologue
AW679099	WS1_22_F04	3.8	3.3	1.5	8.1	2.8	4.0	-1.1	1.8	1.2	3.6	-1.4	3.0	-1.1	e-09	S-adenosylmethionine decarboxylase 2
BF480763	FM1_14_F11	-1.4	-2.3	-2.2	-1.4	-4.2	-2.1	-1.5	1.1	-1.3	-2.0	1.0	-1.3	1.1	e-36	Histone H2B.1
AW678598	WS1_16_B08	-2.6	-1.4	-1.4	-2.1	-1.3	-2.1	-1.1	1.3	1.9	-1.6	1.1	-1.8	-3.1	e-69	DNA cytosine methyltransferase Zmet3
AW677699	WS1_10_H10	-2.8	-2.0	-37.3	-25.1	-2.5	-17.0	-1.2	5.0	3.4	1.7	-3.1	2.1	-1.7	e-22	Chromatin structure regulator
RNA binding proteins/RNases																
CF771804	DSBF1_24_C10	405.7	2.1	1.6	3.6	1.9	7.2	3.3	1.1	-2.3	-3.9	-4.2	-1.6	-2.9	e-20	RNA-binding protein RNP1 precursor
CF756248	DSAF1_4_A06	356.1	ND	3.4	-34.2	-26.7	6.1	5.3	2.9	3.4	-1.4	8.1	1.4	15.3	e-41	Ribonuclease S homolog
BE366362	PI1_32_C05	157.2	1.1	1.0	1.6	1.6	-1.8	4.5	-1.3	-1.9	-4.1	-4.0	-7.5	-12.0	e-43	Ribonucleoprotein
BM324243	PIC1_26_A08	-5.9	-2.9	-3.2	-1.1	1.7	-1.6	-1.3	3.6	2.0	5.9	3.0	2.2	1.3	e-28	RNA-binding protein
Translation																
CF772699	DSBF1_38_C09	9.4	2.3	2.6	10.9	5.5	2.4	5.8	1.0	-1.5	-2.3	-4.1	-4.3	-3.5	e-24	60S ribosomal protein L10
BG465432	EM1_76_D09	5.2	1.8	3.2	3.4	1.1	2.1	3.8	2.0	1.5	-1.1	4.3	-1.0	4.6	e-106	Elongation factor 2 (EF-2)
AW676859	DG1_1_E06	1.1	-2.1	-3.8	-1.5	1.2	-2.8	-2.9	-4.5	-1.1	-3.0	-2.3	-2.0	1.6	e-43	Protein translation factor SUI1 homolog
BG462982	EM1_46_A12	-12.5	-1.7	-1.1	-1.7	-5.1	-4.9	-5.4	2.9	2.3	-2.0	2.3	-1.0	1.4	e-20	EF1-alpha
Protein folding/turnover																
BG411937	OV2_40_A02	385.6	644.3	1029.2	228.9	792.4	30.8	594.5	4.3	-3.5	-7.9	1.6	-1.9	-2.6	e-20	HSP70
AW747586	WS1_73_G10	4.2	4.1	9.3	295.4	120.0	28.3	34.4	11.2	84.3	-1.2	10.5	17.5	47.6	e-39	Heat shock protein 17.2
AW746348	WS1_40_A06	2.3	2.3	1.4	55.7	21.2	2.0	8.9	6.1	15.9	3.1	1.6	2.1	4.6	e-26	Heat shock protein 16.9
CF772585	DSBF1_36_H02	1.5	10.3	82.4	4.2	40.5	38.9	35.6	2.9	1.5	13.1	7.0	21.7	4.7	e-44	HSP70
BI075054	IP1_20_E07	-1.1	5.6	2.6	2.6	319.9	488.8	5.0	10.9	8.2	60.1	9.9	53.5	52.5	e-23	PDI-like protein
BG104091	RHIZ2_40_D08	-1.4	2.0	2.4	1.7	1.5	3.0	2.6	1.0	1.4	-1.1	1.3	1.7	1.1	e-53	Polyubiquitin
Auxin, ethylene regulated genes																
AW679310	WS1_23_D10	3.2	4.0	3.3	4.8	7.1	-1.2	3.2	-1.0	1.0	-1.1	-1.4	4.3	1.5	e-17	Auxin-regulated protein
BE593596	WS1_101_C03	-4.0	2.5	1.1	-1.3	-2.1	3.4	6.3	5.1	3.3	7.9	5.5	6.3	4.9	e-67	Ethylene-responsive protein

[a] S/R is the ratio of basal gene expression between the shoot and root tissue in control, untreated plants.

[b] Positive values indicate gene induction, while repression is represented as a negative value.

[c] cDNAs induced 3-fold or greater are highlighted green, while those repressed 3-fold or greater are shown in red.

[d] GenBank accession number.

[e] Abbreviated EST clone identification.

3 or 27 h with ABA, NaCl or PEG. Collection of tissue after 3 and 27 h of treatment was done to identify genes that responded to the treatments with different kinetics and to minimize the influence of circadian cycling on the results. The results obtained from the sorghum microarray experiments have been incorporated into a relational database accessible to public researchers (Cordonnier-Pratt *et al*., 2004b). Information from a parallel study on changes in sorghum gene expression modulated by SA, JA, ethylene and other treatments is also being incorporated into this database so that gene expression from a compendium of sorghum treatments can be analyzed and cross-referenced (Salzman, *et al*., 2005).

The mRNA levels of ~333 sorghum genes were also quantified in this study using qRT-PCR. The majority of the genes analyzed by qRT-PCR were selected based on results from microarray analysis. However, a number of additional genes were selected from the expanded collection of sorghum EST and methyl filtered sequences created since printing of the microarrays used here (http://fungen.org/Sorghum.htm; http://www.plantgenomics.iastate.edu/maize/). qRT-PCR data was generated because this method has better sensitivity, dynamic range and specificity compared to cDNA microarrays (Rajeevan *et al*., 2001). Moreover, cross hybridization of sequences derived from gene families can occur on cDNA microarrays and incomplete knowledge of the sorghum genome sequence makes this possibility difficult to assess. Therefore, we used microarrays to detect global changes in gene expression modulated by ABA, NaCl or PEG and then carried out qRT-PCR analysis to quantify the kinetics and extent of variation in mRNA abundance for specific genes of interest.

Sorghum genes modulated by ABA and osmotic stress

The mRNA levels of > 1000 genes changed at least 2-fold after plants were treated with ABA, NaCl or PEG in at least one of the time points and tissues examined in this study. Assuming the sorghum genome encodes ~50 000 genes, this indicates a minimum of ~4.1% of the genome is responsive to ABA, NaCl or dehydration. This under estimates the size of the ABA/osmotic stress responsive gene complement in sorghum since our arrays contained sequences from only 12 982 gene clusters and a limited set of conditions, tissues and developmental stages were analyzed. In Arabidopsis, Seki *et al*. (2002a) found ~6.7% of the 7000 genes used to construct full-length cDNA microarrays responded to water deficit or high salinity. Moreover, massively parallel signature sequencing (MPSS) of ABA-treated Arabidopsis plants identified 1354 genes with altered mRNA levels corresponding to ~5.4% of the genome (Hoth *et al*., 2002). Overall, the physical constraints imposed by osmotic stress and the action of the hormone and osmotic stress signaling pathways induced in response to high salinity or cellular dehydration modulate the expression of a wide array of sorghum genes involved in growth, metabolism, transport and protection.

Approximately 27% of the sorghum genes induced by osmotic stress did not show significant homology to previously characterized genes with known or presumed function. The majority of these unknown genes were plant specific and over half were restricted to monocot lineages. Furthermore, 29 of the inducible sorghum genes did not have homologs in the nearly complete rice genome sequence, although homologs of some of these genes could be found in sorghum's closer relatives, sugarcane and maize. Finally, no homologs were found for eight sorghum genes suggesting that they may be sorghum specific. These uncharacterized and potentially novel sorghum genes may provide new opportunities for understanding the unusual drought tolerance of sorghum and the evolution of grass adaptation to water limited and saline environments.

The majority of the genes induced >2-fold in response to ABA, NaCl or PEG treatment had sequences that were related to genes of known or predicted function based on BLAST analysis. Many of these genes were related to genes previously reported as stress responsive in rice, Arabidopsis or other plant species (Kawasaki *et al*., 2001; Seki *et al*., 2001; Bray, 2002; Hoth *et al*., 2002). The identification of these genes in sorghum provides an important baseline of information that will aid follow up biochemical studies of gene function, help identify candidate genes that are the basis of sorghum drought tolerance QTL and enable comparative analysis of osmotic stress responses among grass species. The predicted functions of

the osmotic stress and ABA responsive sorghum genes identified in this study are discussed below.

Genes involved in growth, metabolism, or transport

Exposure of plants to water deficit or increases in root salinity cause rapid inhibition of growth due to loss of cell turgor and/or disruption of water potential gradients (Nonami and Boyer, 1989, 1990; Bray, 1997). Therefore it is not surprising that exposure of sorghum seedlings to high salinity, low water potentials or ABA modified the expression of genes encoding components of the cytoskeleton (e.g., actin, tubulin, actin depolymerization factor, ADF), putative cell wall associated proteins (e.g., glycine-rich and proline-rich proteins), and expansins. This response could be a direct consequence of altered growth rates or may represent adaptive changes in cell wall composition and extensibility. Previous salt stress studies in rice (Kawasaki *et al*., 2001) and water deficit studies in soybean (Creelman and Mullet, 1991) also documented changes in the expression of genes encoding cytoskeletal and cell wall proteins, especially in zones of cell growth (Keller and Cosgrove, 1995). In wheat, differential expression of actin depolymerization factor (ADF) was associated with cold hardiness, a response that increases dehydration tolerance (Ouellet *et al*., 2001). Likewise, the ~30 to >100-fold increase in expression of a sorghum ADF homolog (BE363707) in response to ABA treatment or osmotic stress may play a role in sorghum's response to water limiting conditions (Table 3). Similarly, differential expression of expansins in maize roots has been associated with continued growth of roots at low water potential (Wu *et al*., 2001), an important adaptive response that helps reestablish plant water supply (Creelman *et al*., 1990). In the current study, expression of a member of the sorghum beta-expansin gene family (BG051261) was increased >100-fold selectively in sorghum shoots in response to ABA treatment or osmotic stress (Table 3). In the resurrection plant *Craterostigma plantagineum*, increased expression of alpha-expansins in response to dehydration was correlated with increased leaf wall extensibility (Jones and McQueen-Mason, 2004). It is possible that increased expression of beta-expansin in sorghum shoots in response to osmotic stress may have a similar function.

Inhibition of transpiration, reduction of cell turgor and modification of other aspects of plant water status caused by dehydration and osmotic stress are likely to modify the movement and concentration of ions and other compounds throughout the plant. Moreover, the induction of ion transporters in response to elevated NaCl is an important mechanism used by plants to maintain ionic balance in the plant cytoplasm (Hasegawa *et al*., 2000). In response, expression of a large group of sorghum genes encoding various types of transporters was modified when sorghum seedlings were exposed to elevated NaCl, PEG and ABA. For example, a gene encoding a putative aquaporin plasmalemma facilitator protein (PIP2) was induced strongly by NaCl and ABA treatment in roots at both time points, but more selectively in shoots (Table 3). In contrast, a gene encoding a putative ABC transporter was induced in roots but repressed in shoots. Expression of three genes encoding proteins related to tonoplast transporters was also modulated by these treatments. Several of these genes were more highly expressed in shoots than roots in control plants but were induced in roots following treatment with ABA, NaCl or PEG. Further targeted analysis of specific genes will be required to more fully understand the role of this class of modulated genes in sorghum's response to osmotic stress.

Genes involved in photosynthesis were generally down regulated in shoots following treatment of plants with NaCl, PEG or ABA. This response is consistent with the closure of stomata in response to elevated ABA or osmotic stress, inhibition of carbon fixation and reduced need for energy capture by the photosynthetic electron transport apparatus. The expression of genes encoding pyruvate ortho-phosphate dikinase and malate dehydrogenase, enzymes involved in the C4 acid cycle in sorghum was not modulated significantly in response to osmotic stress. The ortholog of the maize C4 NADP malic enzyme (Tausta *et al*., 2002) was not specifically assayed in this initial survey of gene expression. However, two other genes encoding NADP malic enzyme were assayed and one of these (AW746273) showed a high level of induction in roots and shoots in response to osmotic stress or ABA treatment (Table 3; carbon metabolism). This sorghum gene

is closely related to a maize gene encoding NADP malic enzyme that is highly expressed in the epidermis of embryonic roots (Lopez Becerra *et al*., 1998). In rice, four genes encoding NADP malic enzyme were identified and all were induced in response to osmotic stress treatments to varying extents (Chi *et al*., 2004). NADP malic enzyme has been implicated in fatty acid biosynthesis, regulation of pH and ionic balance, and in the production of pyruvate and reducing power for induction of plant defenses (Drincovich *et al*., 2001). The precise role of this gene in sorghum's response to osmotic stress will require further analysis. Over expression of the sorghum C4 PEPCase in maize improved water use efficiency (Jeanneau *et al*., 2002). Unfortunately, for technical reasons, we were unable to obtain reliable results on the expression of this gene. However, the expression of genes encoding the root and housekeeping forms of sorghum PEPCase was quantified and each gene was selectively modulated by osmotic stress (Table 3).

A group of genes encoding proteins with similarity to lipases, lipoxygenases, choline kinase, and fatty acid desaturases were modulated by the treatments used in this study consistent with membrane modification in response to osmotic stress. Lipids may also be serving as a source of energy under these conditions. Several genes involved in cytidine metabolism were also modulated by the treatments imposed in this study including induction of CTP synthase in shoots and cytidine deaminase in roots.

Genes involved in the protection of sorghum *from abiotic and biotic stress*

One of the largest classes of genes modulated by high salinity, plant dehydration and ABA encode proteins which are involved in the protection of plants from damage associated with dehydration, reactive oxygen scavenging (ROS) and pathogens/insects. Sorghum treated with NaCl, PEG or ABA exhibited enhanced expression of a gene encoding delta 1-pyrroline-5-carboxylate synthase, a rate limiting step in proline biosynthesis (Weinburg *et al*., 1982; Wood *et al*., 1996), consistent with the accumulation of proline in drought-stressed sorghum (Wood *et al*., 1996). In general, induction of this gene occurred between 3 and 27 h of treatment except in shoots of plants treated with NaCl where a 3- to 4-fold increase in mRNA abundance was observed after 3 h of treatment (Table 3). Sorghum also accumulates the compatible solute glycine betaine in response to dehydration (Grieve and Maas, 1984; Wood *et al*., 1996). In plants, glycine betaine is synthesized by the two-step oxidation of choline mediated by the chloroplast localized enzymes choline monoxygenase (CMO) and betaine dehydrogenase (BADH) (McNeil *et al*., 1999). In sorghum, the level of BADH mRNA increased 2- to 4-fold in severely drought-stressed sorghum although this change in mRNA level was thought to be insufficient to explain the 26-fold increase in glycine betaine levels in stressed plants (Wood *et al*., 1996). In the current study, genes encoding CMO (BM318015) and BADH (CD222415) showed relatively small changes in expression in response to ABA, NaCl and PEG treatments possibly because more severe treatments are needed to cause induction of these genes (Wood *et al*., 1996). Sugars including trehalose and polyols also play an important role in osmotic adjustment and protection of plants from injury associated with osmotic stress (Hasegawa *et al*., 2000; Garg *et al*., 2002; Van Dijken *et al*., 2004). Sorghum genes encoding trehalose synthase and trehalose phosphatase were identified in this study, although these genes were not highly regulated in seedlings treated with ABA, NaCl or PEG. In contrast, a sorghum gene with homology to inositol−3-phosphate synthase was induced up to ~30-fold when seedlings were treated with NaCl, dehydration and ABA in both roots and shoots (Table 3). *Myo*-inositol and its derivatives play a very important role in plant responses to osmotic stress and desiccation (Ishitani *et al*., 1996; Loewus and Murthy, 2000). To our knowledge, this is the first time a role for this pathway in sorghum has been implicated in stress responses, suggesting further investigation into the role of *myo*-inositol and its derivatives in sorghum is warranted. Our screen also identified a sorghum gene with homology to a turgor responsive gene from pea that encodes a protein with homology to aldehyde dehydrogenases (Guerrero *et al*., 1990). Interestingly, the plant genes are related to human 'antiquitin', an evolutionarily conserved protein found in the inner ear that is also a member of the aldehyde dehydrogenase superfamily (Tang *et al*., 2002). The exact physiological function of these

proteins in plants and mammals remains to be elucidated.

The dehydrins are a large and important class of proteins involved in plant protection from dehydration-associated injury (Dure, 1993; Close, 1996, 1997). These proteins have been proposed to contribute to membrane and protein stability, metal scavenging and suppression of ROS-induced damage that occurs in plants exposed to high saline conditions or water deficit. In this study, at least 11 gene clusters with sequence similarity to dehydrins or LEA proteins were up regulated in response to ABA, NaCl or PEG (Table 3). Expression of all members of this group of genes was increased in roots and shoots following treatment of plants with ABA. However, the extent and kinetics of the increases in mRNA level in roots and shoots varied considerably among the different genes. For example, genes encoding proteins related to group 3 LEAs were induced to a greater extent in roots treated with NaCl or PEG compared to genes encoding proteins related to *dhn2*. However, sorghum genes encoding dehydrins related to *dhn2* were in general expressed at 10- to 20-fold higher levels in the roots of non-stressed plants. These observations are consistent with the idea that different members of the dehydrin gene family are regulated by an overlapping but diverse set of signaling pathways, *cis*-elements and transcription factors (Buchanan *et al*., 2004). Genes encoding oleosins and dermal proteins were also induced by ABA, NaCl and PEG treatment. Oleosins are best characterized as components of pollen membranes where they are thought to stabilize and alter the permeability of surface membranes (Schein *et al*., 2004). The relatively large induction of these genes in roots and shoots in response to ABA, NaCl and PEG suggests that this type of protein may play a significant role in sorghum's adaptation to water limiting and saline environments.

The closure of stomata in response to high salinity, water deficit or ABA creates a condition where excess energy trapped by the photosynthetic apparatus generates ROS resulting in damage and changes in cellular redox state. The induction of genes that encode proteins related to catalase, superoxide dismutase and ascorbate peroxidase in response to treatment of sorghum seedlings with NaCl, PEG or ABA is consistent with this observation (Table 3). Induction of the group of ROS-related genes occurred in shoots between 3 and 27 h post-treatment initiation. A similar relatively slow induction of ROS-related genes was observed in rice (Kawasaki *et al*., 2001).

Sorghum also modulated the expression of a large group of genes predicted to encode proteins involved in plant defense in response to ABA, NaCl and/or PEG (Table 3, supplemental table IV). A group of genes with similar functions was induced in rice treated with 150 mM NaCl (Kawasaki *et al*., 2001) and in drought-stressed barley (Ozturk *et al*., 2002), indicating that this is a common response to osmotic stress among grass species.

Genes involved in regulation and gene expression

The response of plants to abiotic stress is mediated in part through changes in plant hormone levels, transcription, RNA stability/binding, translation, protein turnover and modification (Hasegawa *et al*., 2000; Zhu, 2001a; Himmelbach *et al*., 2003; Shinozaki *et al*., 2003). The expression of sorghum genes encoding proteins involved in each of these levels of gene regulation was modified by NaCl, PEG and ABA (supplemental tables I and II; Table 3). Interestingly, it appears that there is a significant amount of post-translational regulation that occurs in response to osmotic stress. Specifically, numerous genes involved in protein folding (e.g., heat shock proteins, protein disulfide isomerase) and turnover (e.g., proteosome associated factors, polyubiquitin) were induced by these treatments. This observation suggests that osmotic stress, like heat, affects protein stability and requires action of chaperones and the proteosome to stabilize and/or remove damaged proteins. It is also likely that protein turnover is required to supply amino acids for synthesis of proteins that protect plant cells from dehydration (i.e., dehydrins) or associated biotic stress (i.e., proteins involved in defense).

The signaling network that mediates plant responses to osmotic stress and changes in ABA level is complex, involving numerous protein kinases, phosphatases and signaling compounds such as inositol derivatives and cADP (Finkelstein *et al*., 2002; Xiong *et al*., 2002; Himmelbach *et al*., 2003). Although the current study was limited in scope, genes encoding key regulatory proteins that

mediate responses to osmotic stress were identified and changes in their expression analyzed. For example, a sorghum homolog of protein phosphatase 2C (BM326663), an important mediator of ABA signaling (Leonhardt *et al.*, 2004), was induced within 3 h in shoots by PEG, ABA and NaCl treatment and in roots by PEG and ABA (Table 3). A gene encoding a catalytic subunit of protein phosphatase PP2A−3 (CF772114) was also strongly induced by all three treatments in roots, but up- or down-regulated in shoots depending on treatment and time. Inositol phosphate signaling, cADP and G-proteins mediate many plant responses to osmotic stress (Xiong *et al.*, 2002; Himmelbach *et al.*, 2003). Therefore, it was useful to identify sorghum genes involved in these signaling pathways that are modulated by ABA, NaCl or PEG.

Treatment of plants with NaCl or PEG modulates plant water status and both treatments can cause loss of cell turgor. Plants exposed to water deficit or high saline conditions also accumulate ABA and/or alter the distribution of ABA in plant tissues. The committed step in ABA biosynthesis in plants is mediated by 9-*cis* epoxycarotenoid dioxygenase, and genes encoding this enzyme are induced in drought stressed plants (Schwartz *et al.*, 2003). In sorghum, three genes encoding proteins homologous to this enzyme were identified in the methyl filtered genome sequences (http://www.plantgenomics.iastate.edu/maize/) that were induced within 3 h in response to osmotic stress and ABA (Table 3). This suggests that accumulation of ABA following mild osmotic stress could increase sorghum's ability to synthesize additional ABA. Furthermore, the different sorghum epoxycarotenoid dioxygenase genes were induced to different extents in roots and shoots following NaCl and PEG treatments indicating that the genes are probably responding to loss of cell turgor and additional signals associated with high salinity. Further experiments are planned to test the role of these sorghum genes in ABA biosynthesis and response to osmotic stress.

Plants sense and respond to high salinity through pathways that are somewhat different from pathways that mediate responses to dehydration and ABA (Zhu, 2001b). The differential expression of genes in response to NaCl, PEG and ABA observed in this study is consistent with this idea (Figure 4). Moreover, the influence of the NaCl, PEG and ABA treatments on roots and shoots is likely to differ in terms of how plant water status is modified, and the kinetics and degree of stress imposition. For example, it is likely that NaCl levels increase faster and to a greater extent in roots than shoots following exposure of roots to elevated levels of NaCl in hydroponics. In the early stage of a NaCl treatment, roots may respond to changes in water status and the direct effects of elevated sodium, whereas shoots may respond primarily to a change in water status and signals from roots. This idea is consistent with our finding that changes in gene expression induced by NaCl in shoots significantly overlapped with changes induced by PEG, whereas this was not the case for gene expression modulated by NaCl in roots (Figure 5).

The regulatory network that mediates changes in plant gene expression in response to NaCl, PEG and ABA includes the direct activation of preformed transcription factors by stress signaling pathways and secondary cascades caused by subsequent modification of the gene regulatory network. In the current study, the initial set of genes activated by stress includes enzymes that modify DNA structure directly or indirectly (histones, DNA methylation, polyamines) and a wide variety of genes that encode transcription factors (Table 3). Overall, this study identified and validated the expression of 22 sorghum genes that encode transcription factors that respond to osmotic stress or ABA. These genes encode classes of proteins that are known to mediate responses to ABA and dehydration in other plants (bZIP factors that bind to ABRE *cis*-elements; AP2/ERBP factors that bind to DRE-elements; HD-ZIP; Myb-factors) (Finkelstein *et al.*, 2002; Himmelbach *et al.*, 2003). The expression of many of the genes encoding transcription factors was relatively specific in terms of response to tissue and treatment.

The genes encoding putative transcription factors identified in this study provides a starting point for biochemical testing and validation using knock out mutants (An *et al.*, 2003) or RNAi (Holzberg *et al.*, 2002). The expression data obtained in this study can also be utilized to identify *cis*-elements through analysis of co-regulated sets of genes (Spellman *et al.*, 1998) and through phylogenetic analysis (Buchanan *et al.*, 2004) to further elucidate the connections between

transcription factors and genes that comprise regulons.

Microarray-based analysis of plant responses to ABA and osmotic stress have been performed in Arabidopsis (Seki *et al*., 2001, 2002a, 2002b; Kreps *et al*., 2002; Leonhardt *et al*., 2004) rice (Lin *et al*., 2003; Rabbani *et al*., 2003; Yazaki *et al*., 2004), maize (Kawasaki *et al*., 2001; Wang *et al*., 2003) and barley (Ozturk *et al*., 2002). This study provides a baseline of information that will allow a comparison of the genes modulated by osmotic stress in sorghum, the first representative of a drought tolerant C4 grass to be analyzed by microarray analysis, to those of other plants. Comparison of gene expression and phenotypic responses to abiotic stress among these species is likely to provide insight into the genetic basis of differential adaptation of plants to adverse environments.

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